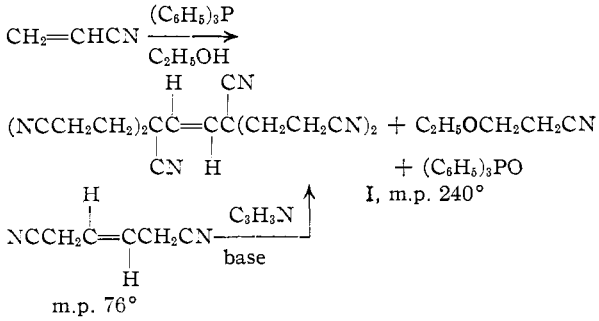
Discussion

While the polymerization of acrylonitrile in the presence of triphenylphosphine produces amorphous high polymer, in the presence of ethanol (and other alcohols), the principal products of the reaction are a high-melting, insoluble hexamer, the addition product of alcohol to acrylonitrile and a small amount of triphenylphosphine oxide.

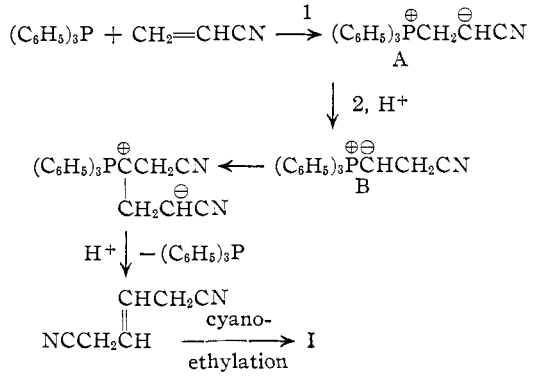


This new hexanitrile, for which we suggest the trivial name of hexacrylonitrile, readily was converted to the corresponding hexacrylic acid II, m.p. 237°, and esters.

The structure we propose is based on (a) the synthesis of the compound in good yield from cyanoethylation of 1,4-dicyano-*trans*-2-butene, (b) molecular weight measurements on the methyl hexacrylate, (c) the infrared spectra which showed no functional group other than the nitrile band at 4.44μ, and (d) a preliminary analysis of the X-ray pattern, which indicated that the molecule must have a center of symmetry.[10]

(10) X-Ray diffraction analysis was carried out independently by Dr. R. E. Hughes (University of Pennsylvania) and Dr. R. R. Pfeiffer (Eli Lilly and Co.). They found the space symmetry to be $P2_1/a$, the dimension of the unit cell to be $a_0 = 15.53$ Å., $b_0 = 6.46$ Å., $c_0 = 9.12$ Å. and $\beta = 107.5°$, and the density 1.20. This requires that there be twelve acrylonitrile units (or two hexamer molecules) per unit cell. Details of this work will be published elsewhere by Dr. R. E. Hughes.

The mechanism of the formation of I presents an interesting problem since one pair of acrylonitrile units must unite in a head-to-head manner. Normally, this would appear to be unlikely in a conventional base-catalyzed polymerization. However, for triphenylphosphine, by invoking a phosphorus ylid intermediate, we can propose a sequence of reactions.



Addition of acrylonitrile to A would produce normal high polymer, as was observed in the absence of ethanol. The proton migration (step 2) would be promoted by a protolytic solvent like ethanol. The stability of a carbanion in an ylid such as B is enhanced not only by the adjacent positive charge but by the possibility of accommodation of the electron by bonding overlap with unfilled 3d-orbitals on the phosphorus.

Since Coyner and Hillman[6] have reported a cyclic dimer of acrylonitrile, 1,2-dicyanocyclobutane, we have prepared a sample and attempted to convert it to I, but without success.

COMMUNICATIONS TO THE EDITOR

CONFORMATIONAL CHANGES ACCOMPANYING AN ENZYME CATALYZED REACTION[1]

Sir:

We wish to report that the catalytic reaction of α-chymotrypsin with diisopropylphosphorofluoridate or *p*-nitrophenyl acetate is accompanied by conformational changes of the enzyme. These conformational changes may have a pronounced effect on the activation of the substrate and therefore make a major contribution to the velocities observed in the enzymatic reactions.[2] Since the hydrolysis of specific substrates for α-chymotrypsin most probably involves the same mechanism as the hydrolysis of *p*-nitrophenyl acetate,[3] this report is of interest for an understanding of α-chymotrypsin catalysis. To our knowledge, conformational changes which accompany a reaction catalyzed by an enzyme consisting of amino acids only, have not been reported previously.

Earlier studies[4-8] demonstrated that the catalytic reaction of α-chymotrypsin with either diisopropylphosphorofluoridate or *p*-nitrophenyl acetate is accompanied by spectral changes of the enzyme. These spectral changes are intimately related to the formation of diisopropylphosphoryl-α-chymo-

(1) This research was supported by grants from the National Institutes of Health and the National Science Foundation.

(2) R. Lumry and H. Eyring, *J. Phys. Chem.*, **58**, 110 (1954); H. Eyring, R. Lumry, and J. D. Spikes in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Eds., The Johns Hopkins Press, Baltimore, Md., 1954, p. 123.

(3) T. Spencer and J. M. Sturtevant, *J. Am. Chem. Soc.*, **81**, 1874 (1959); H. Gutfreund and B. R. Hammond, *Biochem. J.*, **73**, 526 (1959); M. L. Bender and B. Zerner, *J. Am. Chem. Soc.*, **83**, 2391 (1961).

(4) G. P. Hess and J. F. Wootton, *Fed. Proc.*, **19**, 340 (1960).

(5) J. F. Wootton and G. P. Hess, *Nature*, **188**, 4752 (1960).

(6) J. F. Wootton and G. P. Hess, *J. Am. Chem. Soc.*, **83**, 4234 (1961).

(7) J. F. Wootton and G. P. Hess, *ibid.*, **84**, 440 (1962).

(8) B. H. Havsteen and G. P. Hess, *ibid.*, **84**, 448 (1962).

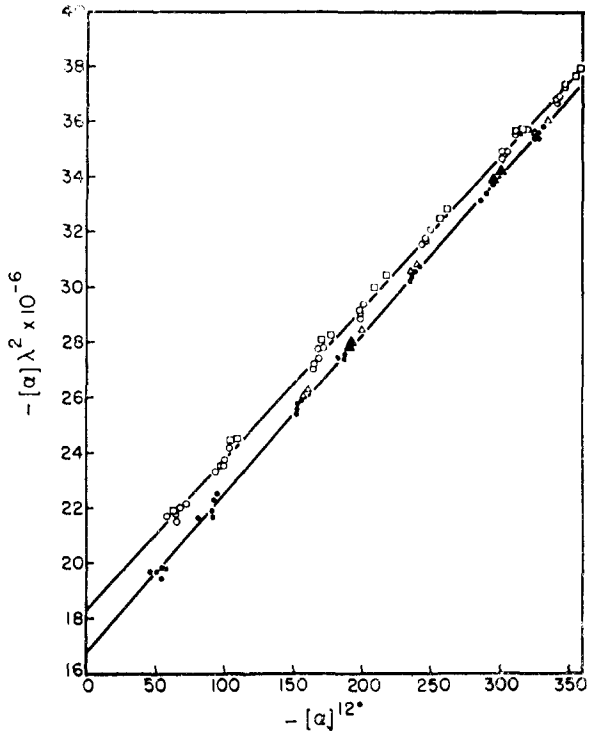


Fig. 1.—Modified Lowry plot of optical rotatory dispersion: α-chymotrypsin, O; diisopropylphosphoryl-α-chymotrypsin, ●; monoacetyl-α-chymotrypsin, △; and the same after deacylation, □; pH 3.8 (acetate buffer, μ = 0.14 *M*), 12°, 2 dm. cell. Protein concentration was 0.08 to 0.15 g./100 ml. The solid lines were computed by the method of least squares using the data for α-chymotrypsin and diisopropylphosphoryl-α-chymotrypsin, respectively.

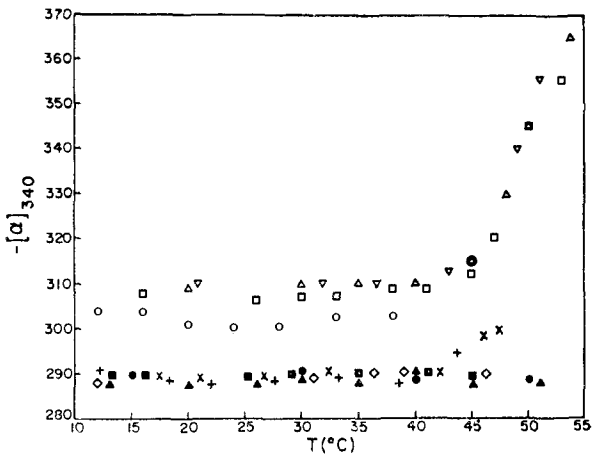


Fig. 2.—The effect of temperature on the specific rotation at 340 mμ.; at pH 4.0 (HCl), 0.10 *M* KCl; concentration in g./100 ml.: 0.11 g. α-chymotrypsin, □; 0.14 g. α-chymotrypsin, △; 0.15 g. α-chymotrypsin, ▽; 0.11 g. diisopropylphosphoryl-α-chymotrypsin, ▲; 0.12 g. diisopropylphosphoryl-α-chymotrypsin, ●; 0.14 g. diisopropylphosphoryl-α-chymotrypsin, ◇; 0.14 g. monoacetyl-α-chymotrypsin (+); 0.15 g. (×); at pH 3.8 (acetate buffer, μ = 0.14 *M*); concentration in g./100 ml.: 0.14 g. α-chymotrypsin, O; 0.14 g. diisopropylphosphoryl-α-chymotrypsin, ■.

trypsin and the formation and decomposition of monoacetyl-α-chymotrypsin. This report is an extension of previous studies[4-8] on the characterization of the spectral changes and concerns itself with the optical rotatory dispersion properties and the effect of temperature on the specific rotation of α-chymotrypsin, diisopropylphosphoryl-α-chymotrypsin, and monoacetyl-α-chymotrypsin.

Three times recrystallized Worthington α-chymotrypsin was used. Monoacetyl-α-chymotrypsin was prepared according to a modified procedure[9] of Balls and Wood,[10] and is a true intermediate in the α-chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate.[9,11] Diisopropylphosphoryl-α-chymotrypsin was prepared using previously published procedures,[7,12] which lead to the specific phosphorylation of α-chymotrypsin. Protein concentrations were determined spectrophotometrically at 280 mμ using a molar extinction coefficient of 50,000.[13] The validity of using the same extinction coefficient for both α-chymotrypsin and its derivatives has been ascertained previously.[3-7] Optical rotation measurements were made with a Rudolph precision photoelectric spectropolarimeter model 80Q.[14] The specific rotations were found to be independent of protein concentration in the range used (0.8 to 1.5 g./l.). The experiments were conducted near pH 4.0 to be close to the pH at which α-chymotrypsin is active without deacylating monoacetyl-α-chymotrypsin.

The modified Lowry plots ($(\alpha)_\lambda = K'(\lambda^2 - \lambda_c^2)^{-1}$) of the optical rotatory dispersion data for α-chymotrypsin, diisopropylphosphoryl-α-chymotrypsin, monoacetyl-α-chymotrypsin, and its deacylated form are shown in Fig. 1. The two lines, representing the data for α-chymotrypsin and for diisopropylphosphoryl-α-chymotrypsin, respectively, were computed by the method of least squares. The intercepts, K', of the two lines differ by twelve standard deviations; the slopes, proportional to λ_c^2, by three standard deviations. The differences in λ_c values for α-chymotrypsin (λ_c = 238 mμ) and diisopropylphosphoryl-α-chymotrypsin (λ_c = 241 mμ) are small. Previously published λ_c values for α-chymotrypsin were found to fall in the 235 to 241 mμ range.[15] There are, however, significant differences in the intercepts of the two lines (-18.4×10^6 for α-chymotrypsin and -16.8×10^6 for diisopropylphosphoryl-α-chymotrypsin). Neurath, Rupley and Dreyer[16] previously have reported a decrease in $(\alpha)_D$ when diisopropylphosphorofluoridate was added to a solution of δ-chymotrypsin at pH 6.0.

The optical rotatory dispersion data for mono-

(9) M. A. Marini and G. P. Hess, *J. Am. Chem. Soc.*, **82**, 5160 (1960).
(10) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).
(11) G. P. Hess and M. A. Marini, *IV Internat. Congr. Biochem. Abstr.*, Vienna, 1958, p. 42; M. A. Marini and G. P. Hess, *J. Am. Chem. Soc.*, **81**, 2594 (1959); *Nature*, **184**, 113 (1959).
(12) E. F. Jansen, M. D. F. Nutting, R. Jang, and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949).
(13) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1957).
(14) We are grateful to Professor Harold A. Scheraga for the use of this instrument.
(15) J. A. Schellman, *Compt. rend. Trav. Lab. Carlsberg, Ser. Chim.*, **30**, 450 (1958); B. Jirgensons, *Arch. Biochem. Biophys.*, **85**, 532 (1959).
(16) H. Neurath, J. A. Rupley, and W. J. Dreyer, *Arch. Biochem. Biophys.*, **65**, 243 (1956).

acetyl-α-chymotrypsin fall on the line established by the measurements for diisopropylphosphoryl-α-chymotrypsin. When monoacetyl-α-chymotrypsin is deacylated at pH 6.0, the resulting molecule has the same optical rotatory dispersion parameters as α-chymotrypsin. This indicates that the changes in the optical rotatory dispersion parameters are reversible and intimately related to the formation and breakdown of the enzyme-substrate intermediate, monoacetyl-α-chymotrypsin.

The effect of temperature on the specific rotation of α-chymotrypsin, diisopropylphosphoryl-α-chymotrypsin, and monoacetyl-α-chymotrypsin is demonstrated in Fig. 2. In the absence of other data $(\alpha)_{340}$ can only be considered as one measure of the thermal stability of the enzymes. For each experiment the same protein solution was used throughout the temperature range. Measurements were made until the solutions became turbid, which occurred at a different temperature in the case of each protein. It can be seen that the specific rotation of monoacetyl-α-chymotrypsin and diisopropylphosphoryl-α-chymotrypsin is considerably less temperature dependent than the specific rotation of α-chymotrypsin.

These experiments demonstrate that the formation of diisopropylphosphoryl-α-chymotrypsin or monoacetyl-α-chymotrypsin is accompanied by significant changes both in the optical rotatory dispersion parameter K', and in the temperature stability of the molecules. Previous experiments[3-7] have demonstrated that the formation of monoacetyl-α-chymotrypsin or diisopropylphosphoryl-α-chymotrypsin is accompanied by spectral changes and in the case of diisopropylphosphoryl-α-chymotrypsin, by increased stability toward unfolding in 8 *M* urea at pH 7.0. These data are consistent with the occurrence of structural changes in the formation of monoacetyl-α-chymotrypsin or diisopropylphosphoryl-α-chymotrypsin. Significant changes in the optical rotatory dispersion parameter K', with only minor changes in λ_c, suggest changes in side group interactions rather than in helical content. While a full interpretation of these data must await a more complete understanding of both the theory of optical rotation and the structure of the enzyme, this conclusion is in agreement with the data presented previously[7] on the characterization of the spectral changes which accompany the formation of diisopropylphosphoryl-α-chymotrypsin or monoacetyl-α-chymotrypsin.

(17) Fulbright grantee, 1959–1961.

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RECEIVED NOVEMBER 2, 1961

THE H_- SCALE AND THE ACIDITY OF AROMATIC AMINES

Sir:

We wish to report the establishment of a self-consistent H_- scale for four systems: water, pyridine–water, sulfolane (tetramethylene sulfone)–water and dimethyl sulfoxide–water, each containing tetraalkylammonium hydroxide. H_- describes the ability of a solvent to remove a proton from a neutral acid and is defined as[1]

$$H_- = -\log \frac{a_{H^+} f_{A^-}}{f_{HA}} = pK_a + \log \frac{[A^-]}{[HA]}$$

H_-, in dilute aqueous solutions, thus becomes identical with pH. The scale was established using the Hammett stepwise technique[1b] with the concomitant determination of the acidities of twenty-three substituted anilines and diphenylamines. Previous work on strongly basic solutions has been reported by Schwarzenbach and Sulzberger[2] (aqueous alkali), Deno[3] (hydrazine–water), Schaal[4] (hydrazine–water, ethylenediamine–water and other systems), and Langford and Burwell[5] (sulfolane–water plus base). However, several discrepancies in both pK_a and H_- exist among their published works. Our results for sulfolane–water containing tetramethylammonium hydroxide are in general agreement with those of Langford and Burwell[5] for the somewhat different system, sulfolane–water containing phenyltrimethylammonium hydroxide.

The acid ionizations were measured spectrophotometrically at 25° and all the indicators listed below showed an instantaneous, reversible, spectral change on addition of base. Those compounds which appeared to have an anomalous ionization behavior were rejected in establishing the scale. In most cases the neutral molecule had little or no absorption at the λ_{max} of the ion. For a given solvent pair variations in the solvent composition had only a small effect on the spectra of the ions.

The compound which bridges the region from dilute aqueous solution to the mixed solvents is 2,4,6-trinitroaniline whose pK_a is 12.20 in aqueous buffers, in good agreement with the value of Schaal.[4b] The pK_a values listed in Table I are averaged values from several solvents (*e.g.*, 4,4-dinitrodiphenylamine; pK_a = 14.09 in pyridine–water–tetramethylammonium hydroxide, 14.15 in aqueous benzyltrimethylammonium hydroxide, 14.00 in sulfolane–water–tetramethylammonium hydroxide). Those compounds which ionize completely in dilute aqueous solution (pK_a values from 2 to 10) have been included because the good correlation existing between acid strength and structure over a range of some 16 logarithmic units helps anchor the scale in the dilute aqueous region. These correlations together with a study of the effects of varying the concentration of hydroxide ion and the identity of the cation will be described in subsequent publications.

Figure 1 shows the variation of H_- with solvent composition[6] for the systems pyridine–water,

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(2) G. Schwarzenbach and R. Sulzberger, *Helv. Chim. Acta*, **27**, 348 (1944).

(3) N. C. Deno, *J. Am. Chem. Soc.*, **74**, 2039 (1952).

(4) (a) R. Schaal and G. Gadet, *Compt. rend.*, **251**, 2176 (1960); (b) R. Schaal, *J. Chim. Phys.*, **52**, 784 (1955); (c) R. Schaal and P. Favier, *Bull. soc. chim.*, 2011 (1959).

(5) C. H. Langford and R. L. Burwell, *J. Am. Chem. Soc.*, **82**, 1503 (1960).

(6) The plots of log $[A^-]/[HA]$ for different indicators against solvent composition were found to be parallel, at a given composition in all cases reported here. Above 60 mole per cent. pyridine, in pyri-